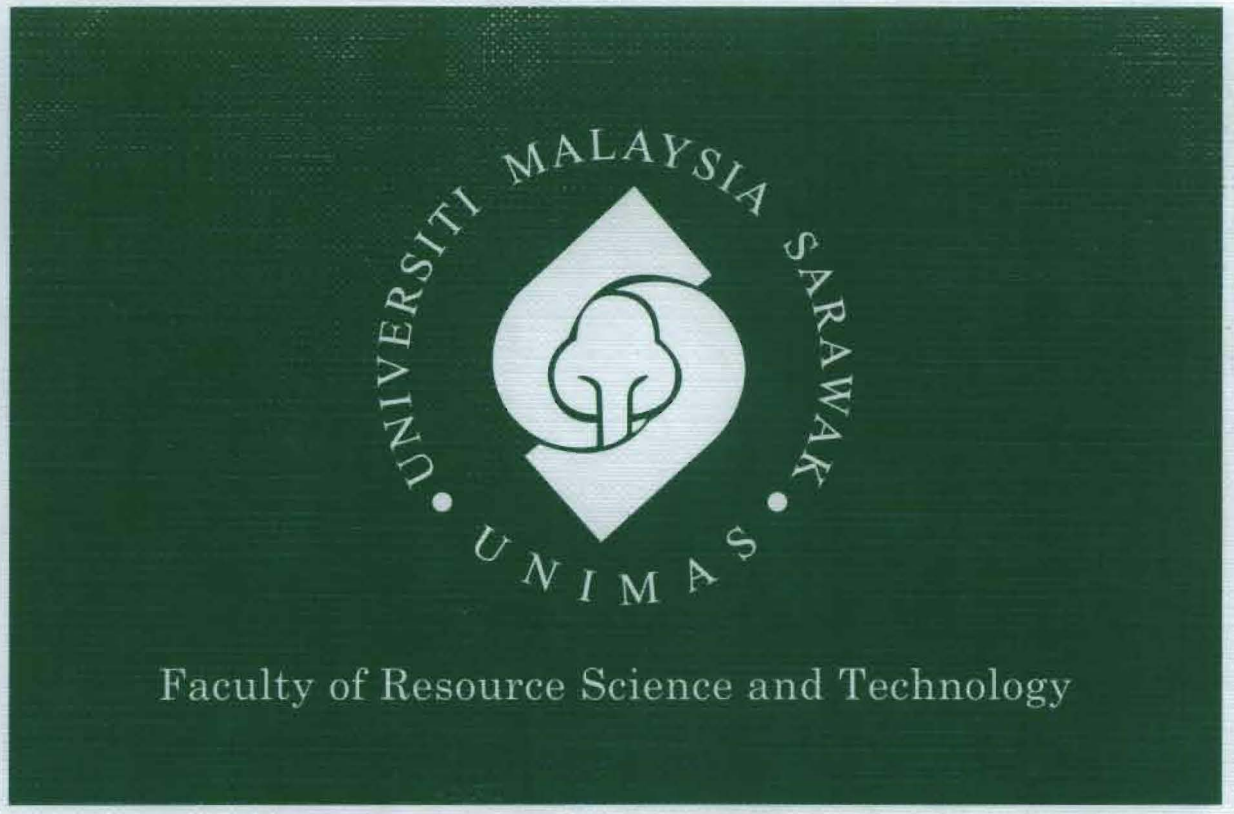

CHARACTERISATION OF MARINE *PENICILLIUM* ISOLATES FROM BAKO, SARAWAK AND THEIR ANTIBIOTICS

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List of Abbreviations

CTAB	Cetyl trimethylammonium bromide
CDA	Czapek dox agar
DRBC	Dichloran-rose bengal-chloramphenicol
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
H_2SO_4	Sulphuric acid
MEA	Malt extract agar
MIC	Minimum Inhibitory Concentration
MHB	Muller hinton broth
MHI	Muller hinton infusion
MDR	Multidrug resistance
NaCl	Sodium chloride
NB	Nutrient broth
OD	Optical density
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PVPP	Polyvinyl Polypyrrolidone
rDNA	Ribosomal DNA
TMP	Sulphonamides and trimethoprim
TBE	Tris Borate EDTA
TLC	Thin Layer Chromatography
UV	Ultraviolet light
WHO	World Health Organization

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Characterisation of Marine *Penicillium* Isolates from Bako, Sarawak and Their Antibiotics

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Abstract

The emergence of multidrug resistant bacteria (MDR) has reduced the efficiency of currently used antibiotics in controlling bacterial infection. Hence, a study was conducted to isolate marine-derived *Penicillium* sp., and subsequently their antibiotics were characterised. Four marine fungi designated as CAS1, CBS1, CCSW and CDSW, were isolated from samples collected from coastal area in Bako, Sarawak. In preliminary screening on Czapek dox agar (CDA) using fungal mycelial discs, the four fungal isolates exhibited strong inhibition zones, which ranged from 17 to 35 mm against *Salmonella typhi* only. Antibiotics extracts from these isolates were then obtained using hexane and dichloromethane (DCM) solvents. In disc diffusion assay, CAS1 hexane extract had strong antibacterial activity against *Enterobacter aerogenes* even at 0.25 mg/ml. DCM extracts of CBS1 and CCSW were active against *Staphylococcus aureus* at 0.25 mg/ml. On analysis using thin layer chromatography, CAS1 hexane extract exhibited four spots with R*f* values 0.17, 0.43, 0.63 and 0.90, respectively. The TLC plates were cut vertically to form individual strips and were used to perform bioautography assay. Through this assay it was found that fractionated components of CCSW hexane extract exhibited strong antibacterial activity against *E. coli*, *E. aerogenes* and *S. aureus*. The DCM extracts of CBS1 and CCSW were active against *E. coli*, *S. typhi* and *S. aureus*. Based on macroscopic and microscopic examinations, all the four isolates were identified as *Penicillium* sp. Molecular identification using primer ITS1/ITS4 identified CCSW as *Penicillium paxilli* (100% similarity). The presence of antibacterial activities with spots that are different from the penicillin-streptomycin control makes it plausible to continue this study with further isolation and characterisation of individual pure compounds.

Keywords: Antibacterial activity, marine-derived *Penicillium* sp., thin layer chromatography (TLC), bioautography assay.

Abstrak

Kemunculan bakteria yang rintang kepada ubat-ubatan (MDR) telah menjejaskan keberkesanan antibiotik dalam pengawalan jangkitan bakteria. Dengan ini, kajian ini telah dijalankan untuk menemui Penicillium sp. jenis marin and seterusnya menguji dan mencirikan antibiotiknya. Empat kulat marin yang kemudian dinamakan CAS1, CBS1, CCSW dan CDSW telah diperoleh pada sampel yang diambil di kawasan perairan Bako, Sarawak. Saringan awal telah dilakukan dengan menggunakan cakera koloni kulat pada agar Czapek dox (CDA) untuk menguji aktiviti antibiotik sampel tersebut. Keempat-empat sampel kulat telah mempamerkan aktiviti antibiotik yang kuat terhadap Salmonella typhi. Ekstrak antibiotik telah diperolehi daripada sampel kulat masing-masing dengan menggunakan pelarut heksana dan diklorometana. Dalam teknik serapan cakram agar, heksana ekstrak iaitu CAS1 telah mempamerkan aktiviti antibiotik yang kuat terhadap bakteria Enterobacter aerogenes walaupun dengan nilai zon inhibisi terendah (MIC), iaitu 0.25 mg/ml. Ekstrak diklorometana CBS1 dan CCSW adalah aktif terhadap Staphylococcus aureus dengan kepekatan 0.25 mg/ml. Seterusnya, analisasi dengan mengaplikasikan kromotografi lapisan nipis (TLC) telah dijalankan. Ekstrak heksana CAS1 mempunyai bacaan faktor retensi 0.17, 0.43, 0.63 dan 0.90 untuk titik pertama sehingga keempat. Kemudiannya, plat TLC dipotong secara menegak kepada kepingan yang berasingan untuk dijalankan ujian bioautografi. Dalam ujian ini, komponen yang telah diasingkan daripada ekstrak heksana CCSW adalah aktif terhadap E. coli, E. aerogenes dan S. aureus. Manakala, ekstrak diklorometana adalah aktif terhadap bakteria E. coli, S. typhi dan S. aureus. Dengan merujuk kepada ujian makroskopik dan mikroskopik, semua pencilan kulat adalah dikenalpasti sebagai Penicillium sp. Identifikasi secara molekul terhadap CCSW dengan menggunakan primer ITS1/ITS4 memberikan keputusannya bahawa pencilan itu ialah Penicillium paxilli dengan persamaan 100%. Kemunculan aktiviti-aktiviti antibakteria pada titik yang berbeza daripada kawalan positif, iaitu penicillin-streptomycin untuk ujian bioautografi menyumbangkan kepada kajian untuk kompaun yang telun pada masa depan.

Kata kunci: Aktiviti antibakteria, kulat marin Penicillium sp., kromotografi lapisan nipis (TLC), ujian bioautografi.

1.0 Introduction

The discovery of penicillin by Alexander Fleming in 1929 has marked the beginning of widespread use of antibiotics in medical field. Consequently, in addition with the vast number of antibiotics being introduced, many formerly life-threatening infectious diseases became curable (Lazzarini *et al.*, 2005). However, after several decades later, the first penicillin-resistant *Streptococcus pneumoniae* was identified in Australia in 1967 (Criswell, 2004). The emergence of these drug-resistant pathogenic microorganisms are evolving and thus rendering the efficacy of penicillin and other antibiotics to low levels (Zhang *et al.*, 2011). In 2004, more than 70% of pathogenic bacteria were predicted to be resistant to at least one of the currently available antibiotics (Vignesh *et al.*, 2011). It is further clarified as World Health Organization (WHO) reported that 95% of *Staphylococcus aureus* worldwide is resistant to penicillin (Kardar, 2005). Increased frequency of antibiotics resistance is posing more challenges in elimination of infection from patients and yet data from WHO shows that 100 million people in developing countries are affected by infectious diseases (Bhadury *et al.*, 2006). Hence, new choices of effective antibiotics should be isolated from natural sources such as marine fungi.

In recent years, marine fungi have been proven to be rich sources for isolation of new natural products with vast range of biological activities (Smetanina *et al.*, 2011). By 2003, more than 272 new compounds have been extracted from marine fungi and this figure is ascending (Tziveleka *et al.*, 2003). These natural secondary products are produced due to their living conditions, salinity, nutrition, high pressure and defense against microorganisms (Liberra *et al.*, 1995). Therefore, the outcomes of these various forms of adaptations may be useful for human beings (Bhatnagar *et al.*, 2010) especially sources for pharmaceutical compounds. Marine fungal-derived compounds such as pestalone have provided new choices

for controlling infectious diseases (Zhang *et al.,* 2009). This compound showed potent antibiotics activity against methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium* (Cueto *et al.,* 2001). Similarly, citrinin, which was isolated from *Penicillium citrinum*, was reported to be broad spectrum antibiotic and especially effective towards elimination of Gram positive bacteria (Devi *et al.,* 2009).

With progressive search for potential natural products from marine fungi, it is predicted that more antibiotics can be isolated from marine environment successfully (Kansoh *et al.*, 2010). In their study, the most active isolate, *Penicillium viridicatum* was shown to produce a broad spectrum antimicrobial agent, fumigaclavine. The work of Liu (2010) has shown that marine environment of Sarawak has the potential to be used as source for isolation of *Penicillium* sp. with antibiotics activity. The long coastal areas of this state prompt further study on marine fungi in the state marine resources.

Therefore, this study was done to isolate antibiotic compounds from marine sources in Bako, Sarawak. The fungal isolates were firstly subjected to preliminary screening for detection of antibiotic properties. Following that, the active compounds in the fungal isolates were extracted using different solvents and the extracts were tested for their antibacterial activities with disc diffusion assay and bioautography assay.

The objectives of this study are:

1. To identify and characterise marine-derived fungal isolates of *Penicillium* sp.
2. To extract and further fractionate antibiotics from most active fungal isolates
3. To detect the antimicrobial activities of selected fungal isolates

2.0 Literature review

2.1 Antibiotics

Antibiotics are antimicrobial compounds which are used to treat infectious diseases caused by microorganisms such as fungi and protozoa (Khan *et al.,* 2010). In 1929, antibiotic was firstly discovered from bread mold *Penicillium notatum* by Alexander Fleming, which is known as penicillin (Criswell, 2004). Since then, there was a blooming of antibiotics production such as methicillin, vancomycin and kanamycin to treat a wide range of diseases.

Antibiotics are categorised based on their modes of action. The major modes of action are namely: inhibition with protein synthesis, interference with bacterial cell wall synthesis, inhibition of cellular machinery and interference with nucleic acid synthesis (Tenover, 2006). The various modes of action are as follows:

a) Antibiotics which disrupt bacterial protein synthesis are tetracyclines, chloramphenicol and oxazolidinones. In addition to difference in structures of bacterial ribosome and eukaryotic cells' counterparts, antibacterial agents selectively inhibit bacterial growth. For example, chloramphenicol binds to 50S subunit whereas macrolides and tetracylines act on 30S ribosomal subunit.
b) The second group include the β-lactam antibiotics such as penicillins and cephalosporins act by disrupting the enzymes which are required for the synthesis of peptidoglycan layer. Glycopeptides such as vancomycin, teicoplanin also interfere with bacteria cell wall synthesis by disrupting the stability of cross-linking peptidoglycan chains.
c) Another group of antibiotics exert their antibacterial effects through interference with the DNA synthesis machinery. For example, Fluoroquinolones cause

breakage of double-strand DNA during DNA replication, whereas sulphonamides block the pathway for folic acid synthesis and thus inhibit DNA synthesis. Moreover, drug combination of sulphonamides and trimethoprim (TMP) blocks the pathway for folic acid synthesis which later inhibits DNA synthesis.

Penicillin was one of the most notable antibiotics discovered as many lives have been saved during second war world (Lewis, 2003). However, its image was tarnished with the emergence of penicillin resistant *Streptococcus pneumonia* in 1967. Since then, antimicrobial resistance has been the major drive in discovery of more antibiotics to combat diseases. An example was the discovery of cephalosporin C from *Acremonium chrysogenum* after the discovery of penicillin (Carolina *et al.*, 2007).

2.2 Antimicrobial Resistance (AMR)

As a consequence of indiscriminate use of antibiotics over time, some bacteria have been evolved to circumvent the effects of antibiotics. In 2004, more than 70% of pathogenic bacteria were predicted to be resistant to at least one of the currently available antibiotics (Vignesh *et al.*, 2011). Some bacteria are resistant to more than one class of antibiotics and hence known as multidrug resistance (MDR). On the other hand, there are bacteria which initially susceptible to certain antibiotic but resistant to it and spread subsequently (Tenover, 2006).

Cases of antibiotic-resistant bacteria were reported throughout the period when antibiotics were first used for treating infectious diseases. Recently, scientists in National Institutes of Health, USA (2012) have discovered an emerging *Staphylococcus aureus* gene, *sasX*, which is responsible in causing methicillin-resistant *S. aureus* (MRSA) in Asia. The

scientists postulated that highly virulent MRSA bacteria affects via horizontal gene transfer (Min *et al.*, 2012). On the other hand, penicillin-resistant staphylococci were reported in 1942 and by late 1960s as well and subsequently more than 80% of hospital and community-acquired staphylococcal isolates were resistant towards penicillin. Furthermore, *E. coli* were reported to be resistant towards cephalosporins and some β-lactams and studies showed that β-lactamase genes were responsible for the resistance (Tenover, 2006).

In addition to that, according to National Institutes of Allergy and Infectious Diseases (2012), various infectious diseases including HIV infection, tuberculosis, influenza, malaria and staphylococcal infection are getting harder to be treated. Patients affected with infectious diseases are forced to stay longer in the hospitals and as well as the need to receive complicated treatments. Thus far, according to Centers for Disease Control and Prevention (2011), antibiotic resistance costs approximately 20 billion annually for health care costs and as well as 8 million more days for patients in the hospital.

Thus far, new antibacterial agents should be discovered progressively for the improvement for human health. In recent years, marine fungi have been proved to be frontier sources for structurally new secondary metabolites with wide range of biological activities. This is due to the fact that they have developed unique metabolic and physiological abilities in addition to their living conditions in natural. Recently, ascending numbers of secondary metabolites were isolated from marine fungi such as quinolinones, scalusamides and shearinins (Mabrouk *et al.*, 2011).

2.3 Marine Fungi as Source of Antibiotics

The marine environment comprises microbes which are yet to be discovered including fungi, bacteria and actinomycetes. Recently, marine fungi are extensively studied for the discovery of antibiotics. Marine fungi can be classified into two groups which are obligate and facultative. Obligate marine are those which thrive and sporulate in marine or estuarine habitat. On the other hand, facultative marine are fungi living in freshwater or terrestrial areas and are able to grow in natural marine environment (Bhadury *et al.*, 2006).

To date, the function of secondary metabolites in the biology of fungal isolates is largely unknown (Fox *et al.*, 2008). Synthesis of these active compounds is postulated to be not required for normal growth of the microbe but may provide other advantages to the organism (Coleman *et al.*, 2011). The most probable function of secondary metabolite in fungi is for survival in its ecological niche. Many fungi species are growing saprophytically, where they are exposed to harsh surroundings and living with various other competing organisms (Fox *et al.*, 2008). In a recent study on defined mutant in the synthesis of active compounds, provides an insight that they are needed for the survival of fungi. LaeA mutant of *A. nidulans*, which had lower level of secondary metabolites, was preferentially consumed by fungivorous arthropod, *Folsomia candida* compared to the wild-type (Rohlfs *et al.*, 2007). Therefore, the secondary metabolites are essential for protection against predation of *A. nidulans*. With that, the outcomes of these various forms of adaptations may be beneficial for human beings especially sources for pharmaceutical compounds (Bhatnagar *et al.*, 2010).

Furthermore, more than 272 new compounds derived from marine fungi have been reported and the number is ascending (Bugni *et al.*, 2004). Vast number of marine fungal

strains were isolated, screened and reported to produce useful antimicrobial compounds. The examples of secondary metabolites isolated from marine fungi are shown in Table 1:

Table 1: Antimicrobial metabolites extracted from marine fungi

Name	Origin	Activity	References
Speradine A	*Aspergillus tamari*	Against Mycrococcusluteus	Tsuda *et al.*, 2003
Citrinin	*Penicillium citrinium*	Antibacterial and antifungal	Devi *et al.*, 2009
Fusarielin E	*Fusarium* sp	Antifungal	Gai *et al.*, 2007
Gliotoxin	*Pseudallescheria*	Antibacterial (*S.aureus*)	Li *et al.*, 2006

More importantly, a number of drugs from marine sources have been clinically approved for pharmaceutical importance such as Tygacil® (Jarvis *et al.*, 2004) and Mycamine® (Frattareli *et al.*, 2004). Furthermore, ascending number of marine derived drug candidates are subjected to clinical trials phases. For example, a microbial drug, Marizomib is undergoing Phase I clinical trials in Nereus Pharmaceuticals, San Diego.

2.4 Discovery of *Penicillium* sp. for Non-Penicillin Based Antibiotics

Emergence of penicillin-resistant bacteria has heightened the isolation of other non-penicillin based antibiotics from this genus. Isolation and identification of antibiotic producing ability of *Penicillium* sp. can be quickly done as they are fast growing species (Bugni *et al.*, 2004). For example, the discovery of secondary metabolites from *Penicillium chrysogenum* includes different penicillin, sorbicillin and meleagrinare (Mohammad *et al.*, 2011). *Penicillium* sp. are known to produce many compounds as they are fast growing species, salt tolerant and also easily obtained from variety of substrates (Bugni *et al.*, 2004).

The work of Kansoh (2010) illustrates how research to isolate marine-derived antibiotic was done. Antibiotic-producing isolate, *Penicillium viridicatum* was isolated from the sediments and sea water of Mediterranean area. Subsequently, this extract from the fungus had high antimicrobial activities against various test bacteria such as *Bacillus cereus, B. subtilis, E. coli* and *S. aureus*. Most significant result was against *B. subtilis* with zone of inhibition recorded 21 mm and it was followed by 19 mm for *S. aureus* (Kansoh *et al.*, 2010). The active compound was then identified through structural elucidation, and found to be fumigaclavine B or its isomer.

Other examples of new antimicrobial metabolites extracted from marine-derived *Penicillium* sp. are shown in Table 2:

Table 2: New antimicrobial metabolites extracted from marine-derived *Penicillium* sp.

Fungus	Compound	Activity	References
Penicillium waksmanni	Griseofulvin	Antifungal	Petit *et al.*, 2004
Penicillium sp *FKI-2140*	Yaequinolones	Insecticidal	Uchida *et al.*, 2006
Penicillium citrinum	Isochromene	Anticoccidial	Smetanina *et al.*, 2007
Penicillium viridicatum	Fumigaclavine B	Antibacterial	Kansoh *et al.*, 2010
Penicillium sp. *SDBF1*	Cysteine Protease Inhibitor	Antibacterial	Mohammad *et al.*, 2011

2.5 Macroscopic and Microscopic Examination

Generally macroscopic examination of colony characteristics is the first step in characterising a fungus and followed by microscopic examination of mycelia and spores. To obtain good samples for microscopic examination, slide culture technique is employed (Harris *et al.*, 1986). A block of agar was used to inoculate fungi and hence the structures of the fungal were then observed. Lactophenol cotton blue was used for staining for the observation of

mycelia structure under light microscope. Microscopic characteristics included mycelia structure, conidia and its arrangement, mycelia fragmentation and sporangia were observed. Besides that, the colours of the spores and presence of septate in hyphae were examined as well.

Alternatively, the slide culture technique reported by Madelin (1969) has also been proven to give a better view of the morphologies of the isolates. Several requirements for a good design for slide culture are met in this technique, including the fungus is easily visible through the cover glass, there is no air space between the fungi and cover glass which will hamper the observation and as well as moist is provided in this chamber to avoid the drying of the fungi (Madelin, 1969).

2.6 Antibiotics Extraction

Extraction is the separation of secondary metabolites from the inactive components by using selective solvents. The concentration and nature as well as the polarity of the solvent will affect the yield and composition of the extract (Ncube *et al.*, 2008). The most commonly used extraction solvents are methanol, ethanol and water (Parekh *et al.*, 2005) and eventually acetone (Nyugen *et al.*, 2011). With the use of increasing polarity of organic solvents and in longer extraction period, a wide range of products can be extracted. Furthermore, the rate of extraction can be further enhanced through shaking.

In a study done by Nyugen *et al* (2011), powdered plant was subjected to extraction for 24 hrs with 100 ml solvent in a glass conical flask on shaker at the room temperature. It was then followed by filtration through Whatman filter paper. The residue was extracted

twice more with each 100 ml solvent subsequently. Finally, the combined solvent extracts was concentrated using rotary evaporator to obtain the crude extracts. Another example of extraction of secondary metabolites was described by Mabrouk *et al* (2011) and in its protocol; extraction was done to obtain the crude extract of antibiotics from *Penicillium brevicompactum.* Both mycelia and filtrate were subjected to ethyl acetate extraction (Mabrouk *et al.*, 2011).

2.7 Antimicrobial Screening Assays

2.7.1 Agar Overlay Technique

There are two methods available for the testing of antimicrobial activities of fungal samples. One of the methods involved spreading the test bacteria on the agar surface and followed by inoculation of the fungi on top of the agar for overnight (Johnson, 1954). Another method is done through growing of fungi on the agar for few days and then the test bacteria are overlaid onto the fungi (Fleming, 1942).

In a study done to test antimicrobial property of *Streptomyces*, Dhanasekaran *et al* (2008) firstly streaked the spores of *Streptomyces* on starch casein agar. Upon incubation for 5 days in room temperature, 10 ml of sterile soft nutrient agar (0.75%) medium was mixed with 0.1 ml test bacterial suspension. Next, the soft NA was overlaid onto the 5 days old *Streptomyces* cultures. The plates were then incubated for 24-72 hrs at room temperature. The zones of inhibition were then detected.

2.7.2 Disc Diffusion Assay

Disc diffusion assay is one of the techniques used to determine the bacterial susceptibility to antibiotics. In this assay, Muller hinton infusion (MHI) agar was utilised for the incubation of test bacteria cultures (Nilsson, 1978). According to a study done by Ines *et al* (2007), the test bacteria were streaked on the respective medium using sterile swab and dried subsequently. Following that, the extracted bioactive compounds were impregnated into sterile filter disk and thus incubation was done at 37°C for 18-24 hrs. Negative and positive controls were included as well for troubleshooting purposes.

In another study done by Pavenden and Rajasekaran (2012), disc diffusion assay was used to determine the antimicrobial activities of *Eugenia singampattiana.* Filter paper discs of 5 mm in diameter were prepared and autoclaved prior to do the experiment. The leaf extracts were then subjected to serial dilutions and added 10 µl to each disc. The sterile impregnated disc with plant extracts were placed on the agar surface subsequently. Negative controls were pre-soaked in solvents. Following that, the plates were then incubated for 72 hrs at 37°C. The zone of inhibition was measured subsequently.

Several factors were reported to affect the reproducibility and accuracy of agar diffusion method. Those factors were thickness and uniformity of the gel, the decision of cut-off size of the inhibition zones and incubation temperatures (Bonev *et al.*, 2008). Besides that, the time of disc application is also one of the considerations while doing disc diffusion assay. The plates which are previously swabbed with test bacteria should not be left at room temperature overtime as multiplication of inoculums may take place. In addition to that, the zone of diameter may be reduced and thus may result in a susceptible strain being reported as resistant (WHO, 1991).

2.8 Thin Layer Chromatography

Thin layer chromatography is used for further separation of the crude extract for identification purposes and to screen the purity of isolated compound (Tarman, 2010). In a study done for the isolation of *Penicillium brevicompactum* from marine fungus, TLC was done for the fractionation of the crude extracts (Mabrouk *et al.*, 2011). Aluminium sheet silica gel 60 was used as the stationary phase and the solvent system was dichloromethane:methanol (95:5) (v/v). Following that, the silica gel sheet was allowed to dry prior to be run in an ascending order for several hours. The resulting spots were located accordingly by their fluorescence on chromatograms under UV light at 254 nm and 366 nm wavelengths. The R*f* values were determined eventually and the purified compounds were used to prepare standard curve. In the results, 11 different compounds were fractionated and two of the compounds were reported active towards *Bacillus subtillis* and *E.coli* (Mabrouk *et al.*, 2011). With reference to another study, spots were sprayed with specific reagents such as vanillin and different acids to reveal the spots in the final step (Nostro *et al.*, 2000). The plate was dried subsequently to produce visible zones.

2.9 Bioautography Assay

Bioautography assay is employed as preliminary screening to detect possible active compounds on the fractionated compounds on TLC plates (Nostro *et al.*, 2000). It is rapid, cheap (Hamburger and Cordell, 1987) and highly efficient as it allows target-directed isolation of active compounds (Rahalison *et al.*, 1991).

There are three types of bioautography assays available namely; direct bioautography, contact bioautography and agar overlay bioautography (Rios *et al.*, 1988). In direct bioautography, after running TLC, test bacteria suspension was spread on the TLC plates and the plates were incubated at 25°C for 48 hrs. Subsequently, microbial growth was visualised with tetrazolium salts such as MTT (Silva *et al.*, 2005). On the other hand, for contact bioautography, the TLC plates will be placed onto inoculated agar layer and left for some time to enable diffusion. The TLC plate is then removed and the agar layer is incubated. The zone of inhibition will be seen on the agar surface where the spots of antimicrobials are previously stuck on the agar (Das *et al.*, 2009). Next, for agar overlay technique, TLC plates will be covered with a molten, seeded agar medium. After solidification and incubation, staining is done to observe the zones of inhibition (Harnorne, 1992).

In a recent study, Shan *et al* (2012) described bioautography assay to detect antibacterial activity of endophytic fungi from *Macleaya cordata.* TLC solvent system 10:1 chloroform:methanol was developed and 5 µl of extracts were sampled onto each TLC plates. Meanwhile, three Gram positive and five Gram negative bacteria were tested against the fractionated active compounds on the TLC plates. These test bacteria suspension was then overlaid on TLC plate on nutrient agar, respectively. Incubation was done at 28°C and 0.5 mg/ml of colour agent, 3-(4,5-dimethylthiazol-2-yl), MTT was sprayed on the TLC plates to visualise the results. In the results, most of the extracts showed antibacterial activity on the test bacteria (Shan *et al.*, 2012). The formation of white inhibition zones against the purple background indicated the antibacterial activity of the separated active compounds (Bernas and Dobrucki, 2000). The diameter of the zone of inhibition of white spot was measured subsequently (Xu *et al.*, 2010).

2.10 Molecular Identification

Morphological characters can be used firstly to differentiate isolates into their phyla and to the correct genera. However, identification to species level is always met with difficulties. Using more methods to identify fungi is always the best solution, because depending solely on one method is highly error prone (Gherbawy and Voigt, 2010). Therefore, with the advent of molecular techniques, the understanding of taxonomic groupings and the evolutionary relationships between fungi can be further enhanced and clarified (Ranghoo *et al.,* 2000). Molecular technology enables the exploration of fungal diversity in a more reliable way (Liew *et al.*, 1998).

Ribosomal DNA (rDNA) has gradually become useful tool for the identification of fungal. The ribosomal gene cluster is consisted of three regions coding for 5.8s, 18s and 28s rDNA genes (Bugni *et al.*, 2004). These tandem repeat sequences are unique among individuals and hence they can be used for identification at several levels (Hibett *et al.*, 1992). ITS regions are very useful in taxonomy. With the utilization of ITS1 and ITS4 primers, ITS1-5.8s-ITS2 region can be amplified by PCR for identification.

Recently, Mohammad (2011) utilized PCR-based identification of marine fungus *Penicillium* sp. from mangrove leaves of *Avicennia marina.* Primers used for PCR were ITS1 (5'-TCC GTA GGT GAA CCT GCG G G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The initial denaturation was set at 95°C for 3min, 35 cycles of 94°C for a minute, 55°C for 1min and 72°C for 1min. It is followed by the final extension at 72°C for 10min. 5µl of the PCR product was subjected to gel electrophoresis and observed under UV illumination for analyses. The results with reference to NCBI Blast reported that the fungi sample was 99% homology to *Penicillium chrysogenum.*